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CLINICAL AND BIOCHEMICAL APPROACHES TO THE STUDY OF LEAD AT LOW LEVELS

Report of a Symposium
February 1970

Summarized by:
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Clinical and Biochemical Approaches to the Study of Lead at Low Levels

While there are current examples of obvious lead poisoning in improperly supervised industrial operations and in pediatric practice where pica for old paint is observed, the health significance of lead compounds in ambient urban atmospheres has not been clearly defined. The lead concentrations in non-industrial atmospheres are very much lower than the current industrial Threshold Limit Value (several $\mu g/m^3$ vs 200 $\mu g/m^3$), and evidence of a specific effect of lead is completely lacking. Nevertheless, it is reasonable to assume that at some point short of clinical lead poisoning in the usual sense, body absorption of lead will reach a magnitude sufficient to cause significant changes in normal physiological mechanisms. That this point is not yet known may be related to the fact that traditional clinical approaches to the detection of lead effects at low levels have been relatively non-specific and incapable of detecting minimal alterations. Nevertheless, it is the question of the existence and significance of minimal alterations or of non-specific effects which is central to discussions of the health significance of the industrial use of lead, its emission, and its consequent appearance in ambient atmospheres, water, and food.

This report contains a summary of discussions held in February, 1970, which attempted to: 1) identify biochemical and clinical approaches to the detection of lead effects at low levels; 2) establish the health significance of whatever lead-related phenomena might be observed at these levels; and 3) suggest possible areas in which research emphasis might be most fruitful in solving the presented problems. Participants included physicians with varying types of experience with clinical lead toxicology, scientists in several branches of clinical medicine and biochemistry, and investigators with primary interests and backgrounds in lead metabolism and experimental toxicology. Several participants, although eminent in their respective fields, had no major interest in lead. They were invited because of their specialized clinical skills and knowledge relevant to organ systems known to be affected by lead.

The material presented in this report reflects the work of the participants, their colleagues, and others in the respective fields of investigation.

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I. Summary of Reports on Environmental and Human Lead Levels

The concentration of lead in soils, foods, water and air is highly variable. The average alimentary lead intake covers a range of at least 0.12 to 0.35 mg/day. There may be a 6- to 10-fold variation between individuals in the average daily exposure to lead from the atmosphere. In spite of the resultant wide range of lead absorption levels, the amount of lead in man is relatively constant, totalling about 100 to 400 mg, of which at least 90% is stored in the bone.

Blood lead levels are even more constant, remarkably so in view of the wide range of exposures. When the mean blood lead levels from various populations are examined, there seems to be a small increase in the blood level as one goes from a rural to an urbanized area. This phenomenon is not well related to the density of motor vehicles in these respective communities, and the urban-rural gradient is observed in areas of the world with a very low number of cars. In addition there is evidence to show that processing of food contaminates it with lead. When one goes from society to society the differences in blood lead levels are not impressive. The blood lead level of rural Peruvians, for example, approximates that for rural North Americans.

Soft tissue lead levels and bone lead levels do not correlate directly in individuals. The lead in the bone seems to increase with age (at least through the fourth decade and possibly beyond), whereas the soft tissue level of lead does not rise appreciably after the end of the second decade. In females the soft tissue lead levels tend to be slightly lower than in males, while in bone the lead concentration in females

is very much lower than in males. This lower bone level of lead, however, also rises with age as in males. Children show very low levels in soft tissue and bone, and no difference between the sexes is demonstrable. Whether the higher bone lead levels in older adults is due to accumulation with age or simply due to the fact that these people were living at a time when there was more lead in the total environment is not known. Lead concentration in bones varies with the bone selected, the lead content being generally higher in the more dense bones.

Bone lead levels have been determined in the skeletons of 40 Indians who lived in Arizona during the period 700 to 1450 A.D. The specimens were from original burial sites and did not include museum material. Soil samples from within the body cavity and adjacent to the buried skeletons contained less lead than the bone; therefore, the question of contamination was excluded. Assay showed 6 to 8 ppm in the bone ash of the rib. Sacrificial skulls from Mexico, probably from the time of the Conquistadors, contained lead levels in the range of 2 to 4 ppm of the ash. Coprolites (mummified fecal material) found with these skeletons give evidence of the diet on which the population lived. The food supply was primarily fish, small grains, and grasses; and the total fecal lead concentration was very low.

At some point in history, environmental and skeletal lead levels apparently rose. Bone samples from the time of the Civil War in this country contain lead in concentrations which approximate those noted in modern autopsy materials, viz., 40 to 50 ppm.

In view of the very great range of lead intake between individuals and variability in lead absorption, the consistency of blood lead levels is impressive, enough so to suggest both regulation by a biological control mechanism and possible biological need. No evidence exists either to support or to deny these possibilities. It is generally true, however, that when a metal is found with similar constancy, the metal is not present merely on an incidental basis but rather as a substance with specific biological importance. In the early studies of zinc biology, for example, zinc was regarded solely as a toxic metal since zinc fumes under appropriate conditions were shown to be responsible for metal fume fever. Zinc was a typical trace metal present in amounts difficult to detect and in association with only traces of information about its significance. When in 1941, zinc was shown to be

an essential constituent of the metalloenzyme carbonic anhydrase, it became apparent that zinc was neither incidental nor solely toxic but in fact had a specific biological function. That a similar situation may apply in the case of lead has never been critically or systematically examined.

In making inferences from blood lead level determinations it is important to remember that the analytical techniques for lead assays at low levels do not yield impressively consistent results. In many situations the variation between replications is greater than the variation between individuals in a sampled population. In addition, the precision of analysis by a given laboratory may vary day to day.

II. Summary of Reports on Lead and Sub-cellular Physiology

The biological function of a number of metals is unknown. The concentration and distribution of these metals in tissues and biological fluids are neither an index of their importance nor of their biological significance, positive or negative. There is still active discussion as to the essentiality and biological function of selenium and chromium; lead may be in a similar category.

Where metals have been known to interact with essential biological systems, they interact with proteins, with nucleic acids, or with integrated systems such as organelles. The proteins that have been examined most extensively are the enzymes. Some enzymes, the so-called metalloenzymes, contain a metal which is bound in such a specific manner that it cannot be removed without loss of activity. A large number of metals are bound to nucleic acids. The function of these metal-containing nucleic acids is presently entirely unknown. The relationship of metals to organelle function is also not understood.

The sites to which metals can attach in biological materials are not infinite. Metals can attach to the epsilon aminoacid group of lysine, to the carboxyl groups of glutamic and aspartic acid, to the sulfhydryl groups of cysteine, and in other less common ways such as to the phenoxy group of tyrosine. In the case of metals such as lead, bonds are stable, immobile, and polydentate. Lead can combine with any or all of these groups; it would be misleading to expect that lead associates only with sulfhydryl groups to form mercaptides.

We do know that lead can be toxic under certain circumstances, but so can any metal. Essential metals often function as components of metalloproteins. When these metals are present in excess and attached to ligands which are not the normal active metal-binding site of these metalloproteins, inhibition occurs, i.e., the metal is toxic. Whether a lead metalloprotein exists and has an essential body function is not known.

Work with carboxypeptidase illustrates several important points. Carboxypeptidase is a zinc-containing enzyme which has two functions: first as a peptidase, secondly as an esterase. It is possible to remove the normal zinc constituent of the enzyme and replace it with various other metals. For example, when the zinc is removed and replaced with cadmium, one finds that the metalloenzyme has lost its peptidase properties, but the esterase properties are increased. Cadmium carboxypeptidase, in fact, is a better esterase than the naturally occurring zinc analog. Lead carboxypeptidase has also been characterized and found to function as an active esterase. The point here is that a lead metalloenzyme could potentially have a perfectly legitimate biological purpose.

Work with this enzyme is instructive in a second sense: It has been found that the metal of carboxypeptidase is attached to three ligands: two imidazolium groups of histidine and one carboxy group. Sulphur-containing aminoacids are not involved. This observation is contrary to many of the expectations which have been expressed in the literature with respect to ligands which might bind lead in natural systems.

Another metalloprotein, metallothionein, was discovered first in the horse, then in other species, and finally in man. Metallothionein is a cadmium-containing protein with a molecular weight of about 6000 without the metal, 6800 with the metal. The protein is composed of some 52 aminoacids, one-third of which are cysteine. According to traditional thinking, this high concentration of sulfhydryl groups should yield a material with great avidity for lead. Indeed, this is easily demonstrable to be so <u>in vitro</u>. However from lead poisoning experiments in animals it has become evident that this is not the case <u>in vivo</u>. In the intact animal lead is not sufficiently strongly bound to metallothionein to displace either cadmium or zinc.

A balance between metals is essential in all considerations of metal biochemistry and toxicity. Metals interact at common sites and thus may be antagonistic, metal A protecting from the effect of metal B, or metal A exaggerating the effect of metal B. An example is that of molybdenum

intoxication which is observed in Somerset in England and in the western part of the United States. The manifestations of this disease may be enhanced by the presence of excess copper. It is quite likely that lead, iron, and perhaps other metals interact in ways which are relevant to this discussion but are not well understood. The competition between metals for ligands depends upon numerous factors, most of which are poorly understood. Included among these factors are the number and character of the ligands, the penetration through membranes by metals, the stability constants for the several metal-ligand bonds, and steric factors describing the spacial arrangement between metals and ligands.

It is relevant to examine systems which might possibly be disturbed by lead and hence represent target systems for lead effects. One such system is the pyruvate-carboxylase system whereby pyruvic acid is introduced into the Krebs cycle by acetyl co-enzyme A. This system is a conglomerate of sulfhydryl-containing enzymes and co-enzymes, including thiamine-pyrophosphate, folic acid, and co-enzyme A. It is essential in the energy metabolism of the brain. We know, for example, that thiamine deficiency, as in Wernicke's syndrome or Korsakoff's psychosis, leads to gross functional abnormalities. A similar syndrome has been observed in foxes fed on fish entrails which contain thiaminase, a thiamine-destroying enzyme. The same clinical manifestations would be expected whether thiamine is destroyed, absent, or its metabolic site of action is otherwise interfered with by metal attachments to relevant sulfhydryl groups. That the central nervous system manifestations of lead poisoning may be related to interference with pyruvic acid metabolism may well be possible. The problem of mercury intoxication, as in the chronic mercurialism of the Mad Hatter, and the question of arsenic toxicity and the therapeutic effect of dimercaptopropanol (BAL), a sulfhydryl-containing compound, are all relevant to the question of metal binding to sulfhydryl groups of the pyruvate-carboxylase system and the disturbance of this system by extraneous metals.

The peripheral nervous manifestations of lead poisoning are not related to these phenomena, but it is entirely possible that lead may attach to sulfhydryl groups of the neuroconduction system peripherally.

Another system where investigation may yield information on the biochemistry of lead is that related to oxidative phosphorylation. This process depends upon the integrity of mitochondrial membranes, which among other things, preserve

the respective levels of calcium and potassium inside and outside the mitochrondria. Impairment of this membrane by carbon tetrachloride is associated with a dramatic shift of calcium into the mitochondria and an uncoupling of oxidation and phosphorylation. The sulfhydryl ligands of the mitochondrial membrane suggest that the attachment of lead or other metals to the membrane may interfere with its intrinsic metabolism and its ability to protect the intra-mitochondrial environment. Under such circumstances the metals might be expected to cause significant damage to the energy metabolism system.

There are undoubtedly ways in which the effects of lead on nucleic acids may be measured. We know that the binding of lead to nucleic acids causes their hydrolysis. Research in this area is currently not well developed, however.

III. Summary of Reports on Lead and the Kidney

Lead in abnormally high amounts may cause acute or chronic renal injury. In children acute lead intoxication may be associated with a renal lesion typical of the Fanconi syndrome. In cases of intoxication without encephalopathy one may see either aminoaciduria or glycosuria or both. In more severe cases, such as with encephalopathy, the triad of the Fanconi syndrome may be seen: aminoaciduria, rickets, and hyperphosphaturia in the presence of extreme hypophosphatemia in the range of 2 mg/100 ml (normal = 3.5 to 4.5 mg/100 ml). Fructose and glucose are excreted in the presence of normal blood levels; however, the ratio between these two sugars varies. Some children excrete close to a gram of citrate in a day although the blood citrate level is normal.

During the acute episode some of these children have shown a picture suggestive of the syndrome of inappropriate secretion of antidiuretic hormone (ADH). This observation cannot be attributed to treatment since it has been observed before treatment is initiated. Treatment itself with EDTA has been shown to cause a transient depression in the serum phosphate levels, and a transient increase in aminoaciduria.

With treatment, many of the elements of the syndrome are promptly corrected. The melliturla is gone within a week; the aminoaciduria clears within a month (semiquantitative paper chromatography). Bone changes resolve within 7 to 8 months.

EDTA mobilization tests in which parathyroid hormone is used as well to mobilize lead stored in the bone have been attempted. In some cases sufficient PTH has been used to

raise the serum calcium level to more than 13 mg/100 ml. Although the amount of calcium in the urine increases 3- to 4-fold, there is no increase in the urinary excretion of lead above that which is associated with EDTA alone.

With respect to chronic renal injury there is, of course, the Australian data which indicate that under certain conditions of exposure lead intoxication can lead to chronic nephritis. Studies in Boston and in Baltimore have failed to demonstrate, however, an equivalent phenomenon in the United States. The EDTA mobilization tests in the Baltimore group were normal, and it was not possible to demonstrate chronic renal injury. Epidemiological factors must account for the dissimilarity between Australian and American experience. In the United States the patients have chewed paint at some time between their first and third year of life. In Australia the children are in the 6 to 10 year age range and absorb lead through the ingestion of raindrops which contain lead from decomposed paint on veranda rails and rooftops. The exposure in this case may be less intense but more prolonged. Many of the Australian patients have a pes cavus deformity and tophaceous gout.

A number of cases of lead poisoning in adults have been observed in Alabama in relation to the prolonged excessive consumption of illegally-prepared whiskey. This disease occurs as well in Georgia, the Carolinas, and indeed may be expected anywhere that whiskey is illegally prepared in soldered vats and distilled through old automobile radiators. There is evidence that over 50 million gallons of illegal alcohol are distilled in this country every year and that over 50% of the illegally-prepared alcohol contains lead at concentrations of more than 1 mg/l. Lead concentrations of 20 to 50 mg/l. and, rarely, even as high as 80 mg/l. have been reported.

Renal disease has been observed in a number of these persons exposed to lead-contaminated illegal alcohol. The extent to which lead is the specific cause for the renal disease is not established. In Alabama experience the typical patient is a negro male between the ages of 45 and 60. Perhaps some 25% of hospital admissions in this group have been exposed to illegal alcohol. Those who have experienced severe and prolonged exposure of 10 years or more are typically found to be anemic, to have a stable renal insufficiency, and to show a normal pyelogram. The kidneys, while symmetrical, are occasionally somewhat smaller than might be expected. There

is no evidence of infection or of glomerulonephritis. The urine sediment is nonspecific with very few cells, and the level of protein excretion less than 1 g/l. Glycosuria is absent. Renal biopsy shows interstitial fibrosis without inflammation. Glomeruli are rather well preserved; however, there is evidence of degenerating tubules and a loss in the number of tubules. Most of the patients have nephrosclerosis of varying degrees as well.

Patients in this group with long-standing lead exposure and renal impairment may have symptomatic saturnine gout, so-called because of its association with lead absorption. This syndrome can be differentiated from primary or hereditary gout by the fact that a) the onset of gout follows the onset of renal disease, b) there is no increased pool of urate, c) the clearance for urate and urea is reduced out of proportion to decreasing creatinine clearance, and d) an anemia is invariably present. In primary gout there is an increased metabolic pool of urate, and renal injury often follows rather than precedes the appearance of clinical gout. All patients thought to have lead nephropathy have been without exception severely dosed over long periods. That lead under different circumstances may either increase or decrease the excretion of urates may reflect a variable disturbance in the renal handling of urate and shifts in the balance between active resorption and excretion of this solute.

The classical hallmarks of lead nephropathy are nephrosclerosis, advanced tubular degeneration, interstitial fibrosis, and intranuclear inclusion bodies. The bodies are acid-fast inclusions which do not contain iron. They are unlike inclusion bodies seen in viral diseases which affect the kidneys, particularly cytomegalic disease.

Considerations of clinical renal disease caused by lead, particularly the relatively acute episodes in children, suggest possible physiological interpretations. The acute effect of lead upon the kidney is manifested by a disturbance of transepithelial transport mechanisms for a variety of seemingly unrelated solutes: glucose, aminoacids, phosphate, and urate. In the normal kidney these solutes are resorbed in the renal tubule, against a concentration gradient, by an energy-dependent, energy-generating transport mechanism. In the lead syndrome the transport of each of these proximal tubule resorbed solutes is modified, giving rise to the constellation of abnormalities which constitute the Fanconi syndrome. It is known from studies of intestinal epithelium and isolated perfused nephrons that the transport of glucose is in some

way sodium-dependent. Studies of isolated bacterial systems show that the transport of some aminoacids can be sodium-dependent. There are also reasons to believe that phosphate transport is sodium-dependent. All these presumably sodium-dependent transport mechanisms then are impaired by lead. Simultaneously, uric acid is moved from the capillaries into the tubular fluid. The impairment of uric acid transport by lead results in a retention of uric acid and uricemia. There are reasons for believing that uric acid transport may be sodium-dependent. The mechanism of excretion of lead is not fully understood, but at least in the chicken it involves the passage of lead from the capillary across the cell to the tubular lumen. Lead is also filtered so that there may conceivably also be a reabsorptive mechanism for lead. In any case, lead crosses cells and in this transit may alter the machinery for the transport of glucose, aminoacids, phosphates, and urates, the mechanisms for which appear to be closely coupled to sodium transport.

Sodium transport is the principal energy consumer in the kidney, accounting for at least 50% and perhaps 70 or 80% of oxygen and substrate consumption. The search for a common denominator for these various transport abnormalities suggests that one examine the sodium transport and the energy production and utilization mechanisms in the kidney.

A reasonable hypothesis is that lead in transit through the epithelial cell modifies the structural and functional integrity of mitochondria. We know that mitochondria are well equipped for the transport of calcium, and it is quite possible that lead is transported in the same way. Lead within the mitochondria might well modify the energy-producing mechanisms which drive the solute-transport mechanisms for glucose, phosphate, aminoacids, and urate. In view of the evidence that these transport mechanisms are coupled to sodium resorption, it would be most surprising to find that a defect in sodium transport is not present as well when these lead-induced modifications are manifest.

Definitive studies to examine this hypothesis have not yet been conducted. Experiments have been conducted, however, which shed light on several features of the functional renal impairment due to lead. When rats are fed for ten weeks on a diet containing 1% lead acetate, the total urinary aminoacid nitrogen increases to approximately 2 to 4 times normal. If the renal tubular cells had been killed, as with cadmium, the excretion of alpha amino nitrogen then would be approximately

20 times normal. One can therefore infer that in the lead experiments the renal tubular cells are functioning but at a less than optimal level.

When one does clearance studies, the clearance of glycine is not increased to the extent of that for the other amino-acids; however the amount of glycine is very much increased in the urine. This reflects a marked increase in the plasma content of glycine, an overflow kind of aminoaciduria for glycine. One wonders whether this glycine comes from an impaired conjugation of glycine and succinate in the synthesis of d-amino levulinic acid, a question raised by Haeger-Aronsen years ago. The clearance of tyrosine and histidine is increased more than that for the other aminoacids. Since lead can form bonds with the phenoxy group of tyrosine and the imidazolium group of histidine, one can speculate that the binding of lead to these two aminoacids is related to their clearance.

The aminoaciduria may be related to changes in mitochondria. Isolated mitochondria have been studied and show swelling and a defect in oxidative phosphorylation. This defect is demonstrated with a pyruvate substrate but not with succinate. These findings indicate that lead interferes with early stages of electron transport, this interference being incomplete and not blocking electron transport entirely. Additional studies are required to localize the effect of lead, but preliminary studies suggest that it occurs at "complex I" or the NADH-coenzyme Q reductase complex, the first part of the electron transport system.

Mitochondria from normal kidneys have been isolated and maintained in a medium with pyruvate-phosphate buffer, no EDTA, and various concentrations of lead. At a lead concentration of 1×10^{-5} M, there is a small effect with partial uncoupling. At a concentration of 2×10^{-5} M there is complete paralysis of respiration indicating a toxic effect of the lead upon kidney mitochondria. The concentrations of lead affecting kidney mitochondria are much lower than those known to be toxic in the whole kidney. It is not possible or appropriate to compare the effects on respiration of similar concentrations of lead in mitochondria from *in vitro* studies to *in vivo* studies. Nevertheless, similar concentrations in both systems appear to cause respiratory impairment of the same order of magnitude.

Studies have been conducted to identify the sites of lead localization in organelles. In control rats more lead is in the cell nucleus than in the mitochondria; however for those rats on 1% lead acetate diets there is a large increment

of lead in the nucleus, primarily in the intranuclear inclusion bodies. Concentration of lead in the intranuclear inclusion body can be demonstrated by autoradiographic technique following exposure to labelled lead and by electron microprobe analysis.

The morphological appearance of the intranuclear inclusion body in rats is the same as that observed in humans acutely exposed to lead. The electron microscopic appearance of the body is characteristic. There is a dense central core surrounded by a periphery of fibrillar material containing some kind of material in the matrix. The nucleolus in the nucleus is normal under these conditions indicating that there has been no inhibition of protein synthesis. It is believed that the formation of the intranuclear inclusion body is independent of the nucleolus and that the body is formed by accretion of lead-complexed protein and grows by continual addition of this material. Intranuclear inclusion bodies occur typically only in the nuclei of the proximal tubular cells. Pseudoinclusions consisting of laminar figures representing some lysosomal phenomenon are commonly seen in the cells of the distal tubules in lead poisoning but are not specific for this form of cellular injury.

The intranuclear inclusion body may be isolated by the classical methods for isolating nuclei and nucleoli. Subsequently ultrasonification destroys the nucleoli, but does not damage the intranuclear inclusion bodies or alter their morphology. Approximately 5 mg of material consisting mostly of intranuclear bodies can be obtained from sixteen rat kidneys. When the bodies are treated with RNA-ase or DNA-ase there is little if any effect, indicating the presence of minimal amounts of RNA or DNA or their complete absence. Treatment with proteolytic enzymes such as trypsin does cause a change which apparently reflects the splitting off of a protein fragment. The remaining materials presumably still contain lead and some protein. More positive identification has not been possible with the quantity of material available.

There is evidence that lead enters the urine by both glomerular filtration and by secretion across tubular lining cells. It is hypothesized that lead transported across the tubular lining cell is in the form of a diffusable ligand complex and that mitochondria are exposed to this complex. Lead in the urine has been shown to be in both an inorganic and an organic form. The organic form is a complex with a small ligand which has not been positively identified. In

cases of lead poisoning, it is apparently the organically complexed lead which increases in the urine.

Intranuclear inclusion bodies may represent a storage of excess quantities of lead that enter the nucleus of the proximal tubule cell. Bodies are noted in rats when their drinking water contains lead at a concentration of 0.4 mg/ml (400 ppm), a level of exposure in the adult rat which does not cause anemia or other signs of detrimental effect.

It has been noted, however, that effects can be observed at this and lower dose levels if rats are placed on a low-calcium diet, i.e., 0.1 g/100 g, which is just sufficient to prevent hypocalcemia. On the low-calcium diet, lead at a concentration of 0.2 mg/ml (200 ppm) has to date led to abnormalities typical of clinical lead poisoning including elevated d-ALA and blood lead levels.

Concentrations of lead in drinking water to which rats show no significant response are still relatively high. This suggests that the rat is a relatively resistant species to lead intoxication. The rat may serve, however, as a model to study the various factors which influence lead intoxication, such as low-calcium diets or pre-existing renal disease.

It is possible to develop an hypothesis which relates solute transport failure, disturbances of oxidative phosphorylation, and lead in the intranuclear inclusion bodies. Central to this hypothesis is evidence of the trans-tubular secretion of lead across the proximal renal tubule. Although 95 to 99% of blood lead is attached to the red blood cell, there is a ligand-bound diffusible form of lead which passes across the renal tubular cell. Mitochondria are exposed to this lead, which moves against a gradient into the nucleus and into the intranuclear inclusion bodies, probably as a lead-lipoprotein complex. In a sense, the inclusion body represents a protective mechanism tending to withdraw lead from the cytoplasm of the renal tubular cell.

If one is to examine the effect of lead on oxidative phosphorylation, it is useful to examine experiments which have been done in cases of experimental carbon tetrachloride intoxication. When this chlorinated hydrocarbon is administered to rats and oxidative phosphorylation is examined with succinate, glutamate, and octanoate substrates, one observes that uncoupling does not occur until some 10 to 12 hours after administration of the carbon tetrachloride. There is then rapid uncoupling which exists for some 20 hours, after which

time the process returns to normal. It is easy to uncouple oxidative phosphorylation with lead *in vitro*, in which cases the mitochondria show a high increase in lead content. When *in vivo* studies are conducted, however, the mitochondria, while showing a very high concentration of lead as compared to all other subcellular fractions, show no evidence of impairment of oxidative phosphorylation.

In some way which is not understood the intact animal manages to compensate and adjust for the lead insult. One might suspect that in the intact organism lead would have a better opportunity to attach to differing ligand sites than would be the case in *in vitro* experiments. *In vitro* experiments, in which the effect of the order of addition is examined, often show that the addition of substrate and co-factors influences the acessibility of binding sites on enzymes to administered metals. The inhibition of lipoic dehydrogenase by lead, for example, is markedly affected by the presence of coenzymes and substrates. Presumably in the living animal in which combinations of co-factor, substrate, and enzyme represent the usual condition, lead might have a better chance to interfere. That appears to be highly variable, however, and each enzyme system of interest requires separate study.

Additional clinical and experimental studies relevant to the effect of lead upon renal function might be attempted:

1. A group of patients with lead intoxication might be put on a salt-free diet. It is conceivable that the distal tubule and the loop of Henle might not be able to compensate for a defect in proximal tubular sodium resorption. A simple screening technique might test and demonstrate a defect in proximal tubular resorption of sodium. If the test is negative, it is not meaningful; if it is positive, however, it is highly significant.

2. Tubular micropuncture techniques might be used to demonstrate in rats whether or not there is a defect in proximal tubular resorption of sodium.

3. Sodium transport in kidney tissue slices can be studied by *in vitro* techniques now available.

4. Uric acid is presumably transported by the same system that transports organic anions. The kidney uses as a primary

energy substrate fatty acids, which are delivered primarily by organic anions, e.g., paraaminohippurate. One can measure how effectively rabbit kidney cortex slices concentrate paraaminohippurate from solution in vitro and the influence on the process of lead added to the medium.

5. Kidney cortex slices may also be used in a similar study of the uptake of aminoacids.

6. ATP levels in the kidney and levels of substrate and co-factors associated with glycolysis and oxidative phosphorylation can be examined.

7. The examination of oxidative phosphorylation in the mitochondria of lead-poisoned animals might be considered.

8. Aminoacid infusions might be examined in lead poisoning to see if an aminoaciduria ensues in any predictable manner.

9. Work on the renal transport of lead should be continued.

10. Studies of sodium transport in the isolated frog kidney, red blood cell, and other physiologically active tissues are appropriate.

11. The in vivo kinetics of glucose resorption in the presence of lead can be examined by glucose titration studies. Bicarbonate titration studies can be utilized to test this sodium-linked proximal tubular function.

IV. Summary of Reports on Lead and the Hematopoietic System

Discussions on the hematopoietic effects of lead focus to a major extent upon disturbances of heme synthesis. As heme synthesis occurs in virtually all cells and is essential for normal cellular function and structural integrity, a review associated with the hematopoietic system broadly overlaps with other areas, particularly those related to the nervous system.

It has been known for years that lead can induce changes in the mature red cell in vivo, in vitro, and in studies whereby red cells are exposed to lead in vitro and returned to the

living organism. Anemia due to lead has been described in children and in adults. In lead workers on the job significant anemia is rather rare, perhaps noted in 2 to 3%. In hospitalized lead workers and children, however, the majority have anemia. In children the effects of lead are often combined with iron deficiency. The anemia is typically microcytic and hypochromic; however it is sometimes normocytic and normochromic. The reticulocytes are usually elevated to a minor degree, perhaps 2 to 12%. There is an increased erythrocyte index, meaning increased erthyropoietin activity or at least increased stimulation for red cell production. There is no evidence that a G6P deficiency, such as is observed in some 10% of American negroes, has any effect on anemia due to lead. Similarly, alcohol does not appear to influence the prevalence or degree of anemia noted in lead workers.

Studies of lead workers show that red cell survival may be shortened in some instances. In hospitalized patients, however, the proportion with decreased erythrocyte life spans increases to about 50%. In children the shortest survival time is associated with the shortest and most acute lead exposure. With intense, acute exposures an acute hemolytic anemia may be seen. Some years ago patients with carcinoma, who were treated with lead because of a presumed anti-neoplastic effect of lead, were observed to have acute hemolytic anemias.

Ferrokinetic studies in lead poisoning have demonstrated that iron is absorbed from the gut, transported, and bound normally. The transfer of iron into the developing red cell does not seem to be impaired. In vitro studies, however, show that high concentrations of lead seem to interfere with the passage of iron from transferrin to the developing red cell. Long chronic exposure to lead appears to reduce the turnover of iron, the clearance of iron from the plasma, and the utilization of iron for new red cell production. In acute exposures in children ferrokinetic studies are typical of what is seen in hemolytic anemias. There is a rapid uptake of iron in marrow, a rapid clearance of iron from the plasma, and a rapid utilization of iron for red cell production.

Stippled cells have been classically recorded in lead poisoning. There is no correlation known between their number and the intensity and type of symptoms observed. Experiments in animals suggest that stippled cells are preferentially sequestered in the spleen. Splenectomy or administration of lead to splenectomized animals results in an increase of number of stippled cells. The spleen reduces the number of

stippled cells either by maturing them and returning them to the systemic circulation or by destroying them.

The lead-poisoned red cell is brittle and inelastic. Its decreased osmotic fragility or increased resistance to osmotic stress is undoubtedly a membrane effect. ATPase activity is decreased in the red cell membrane. Permeability to cations is increased; potassium, especially, is lost through the membrane. It is likely that these observations are related to modifications in osmotic fragility. The Coombs test may be positive, particularly if blood is permitted to settle and the test is done on the younger red cells at the top of the column, presumably because transferrin and other globulins may be retained on the cell membrane of reticulocytes.

Electron microscopic studies of normoblasts reveal a number of changes characteristic of lead intoxication:

1. Gaps in the membrane of the normoblast nucleus are increased both in number and in size.

2. The protein-secreting Golgi apparatus is tremendously dilated.

3. The mitochondria are enlarged. The crystal poles are pushed apart.

4. Myelin bodies are observed. These are electron-dense bodies between the nuclear membrane and the mitochondria.

5. In a variable proportion of nucleated red blood cells, mitochrondria containing non-heme iron are arranged in a ring about the nucleus (ringed sideroblast). The iron is present as molecules of ferritin and as ferruginous micelles. The chemical nature of the fine, dust-like ferruginous micelles is unknown; they constitute an abnormal form of intracellular iron. Similar ringed sideroblasts are found in primary sideroblastic anemia, in pyridoxine-responsive anemia, and in a wide variety of secondary sideroblastic anemias. The pathogenic mechanism common to all seems to be a defect in heme synthesis.

Special studies show the reticulocyte to have a markedly irregular outline and to contain numerous mitochondria heavily laden with non-heme iron, the ferruginous micelles. Stippling in animals and patients is characteristic of lead poisoning. The cause of the stippling is not understood, but the stipples

themselves are composed of altered aggregated ribosomes. In the polychromatophilic cells the polychromasia is due to the retention of ribosomal material. In lead poisoning the incorporation of ^{32}P into phosphatidic acid in mature red blood cells is decreased.

When lead is administered subcutaneously over a period of time in sufficient concentrations, a subcutaneous granuloma forms, a "plumboma," the presence of which may be associated with plasmacytosis and hypergammaglobulinemia.

For some time it has been known that protoporphyrin, coproporphyrin, and d-ALA increase in the red cell in the presence of lead intoxication. With improved isolation of individual enzymes it has become possible to examine the effects of lead on the various stages of the heme biosynthetic pathway. It is clear that lead affects multiple sites at concentrations of 10^{-5} to 10^{-3} M. The main effects are early in heme synthesis at the formation of d-ALA, and later at the point at which iron is inserted into the protoporphyrin molecule by heme synthetase. These processes take place within the mitochondria. More recently it has been shown that ALA dehydrase is inhibited as well.

Practically every living cell contains heme; these disturbances of heme formation are relevant not only to blood production but equally important to cytochrome formation as well. The kidney contains large amounts of ALA synthetase as does the liver. Drugs which induce ALA synthetase in the liver can produce enormous concentrations of this enzyme there, and in the kidney as well.

Acute intermittent porphyria (AIP) is in some respects a suitable model for the study of certain aspects of acute lead intoxication. This inborn error of metabolism tends to occur in families, most commonly in women of the 20 to 30 year old age group. Presenting symptoms include acute unexplained abdominal pain, vomiting, constipation, and neuropsychiatric manifestations which encompass a broad range of phenomena from motor weakness to psychiatric disturbances. Signs include tachycardia, hypertension, decreased reflexes, and sensory abnormalities. Concentrations of d-ALA synthetase, d-ALA, and porphobilinogen (PBG) are increased in the blood. ALA and PBG are elevated in the urine as well. Asymptomatic relatives of persons with acute intermittent porphyria may show only the biochemical alteration of this disease, viz., increased urinary ALA and PBG.

Other porphyrins which may be found in the urine on laboratory examination are not excreted but rather are formed in the voided urine as degradation products.

Isolated and purified ALA and PBG show no evidence of pharmacological activity; they are not pressor substances. The clinical phenomena associated with AIP can be explained on the basis of a disorder of the nervous system. Indeed it has been shown that the peripheral nerves of patients with this disease can undergo demyelination. The presence of macrophages containing lipid-staining material around the nerve gives evidence that these changes occur ante-mortem. Brain demyelination has been noted around vessels. The inappropriate secretion of ADH has been noted in the presence of changes in the thalamic region.

With lead a similar symptom pattern is observed: abdominal pain, vomiting, constipation, and peripheral nerve disturbances primarily of motor function. Anatomically, it has been shown that lead has an effect on myelin sheaths, Schwann cells, anterior horn cells, and axons. The pattern is strikingly similar to that in AIP.

In AIP the main biological disorder is found in the liver, which shows a high level of ALA, PBG, and ALA synthetase. There is reason to believe that an association exists between the hepatic biochemical alterations and the morphological brain changes. Lead appears to act directly on the brain and nervous and hematopoietic systems. Thus, it would appear that AIP and acute lead poisoning have different basic causations but show a final common pathway of clinical manifestation.

In the search for more subtle indices of lead effect, examinations have been conducted on lead levels and heme precursors in children. When lead is moderately elevated in children into the 40 to 60 μg/100 g range one notes a relatively poor correlation between the blood lead level and ALA, coproporphyrin, and other heme precursors. This contrasts somewhat with the situation in industrial workers, for whom abnormal concentrations of these materials are almost invariably found at the 60 μg/100 g range. In studies of groups of normal children and those with mental retardation reflecting a variety of etiologies, a difference in serum ALA between the two groups has not been observed. Similarly, there has been no difference in blood lead levels nor in the lack of correlation between blood lead and intelligence, or between ALA dehydrase and intelligence. A significant correlation has been found, however, between blood lead levels in the 40 μg/100 g

range and ALA dehydrase in the blood. The clinical significance of this correlation is not established.

There is experimental evidence for a correlation between ALA dehydrase levels in liver, brain, and blood. Such evidence is derived from studies on suckling rats who have received lead only through the milk of their mothers, who had been fed on a 5% lead acetate diet after the litter had been delivered. Neuropathological changes were observed in the suckling mice under these circumstances. The clinical implications of this work are obviously not fully defined inasmuch as interrelations between lead, depressions of blood and brain ALA dehydrase, and anatomical changes of the central nervous system are not well understood.

In summary, however, it has been shown that (1) ALA dehydrase activity is reduced in both the blood and brain of lead-poisoned rats, (2) a significant correlation exists between blood and brain ALA dehydrase activity in normal and lead-poisoned rats, and (3) a correlation exists between blood lead and ALA dehydrase levels in children. It is possible that diminution in ALA dehydrase activity in the peripheral blood of children may reflect a similar reduction of the enzyme in the brain. (The presence of ALA dehydrase in human brain may be assumed since positive enzyme activity was found in the brains of two aborted fetuses which were at the 15th and 17th week of gestation.) Thus, it is likely that a negative correlation exists between the blood lead level in children and the ALA dehydrase activity in the brain. These observations suggest that it is appropriate to consider seriously the possible effect on the developing brain of lead levels previously regarded as normal.

Preliminary studies of lead-poisoned dogs have been conducted to shed light on the physiological significance of measurements which show a depression of ALA dehydrase. In such studies dogs were fed for 42 weeks on diets containing 0, 100, and 500 ppm of added lead. At the end of the 42-week period the blood ALA dehydrase level was virtually zero in the group of dogs fed 500 ppm lead. However, the behavior and appearance of health in all animals was the same. No animals appeared to have been adversely affected by the ingestion of lead over this period. Although these animals did not appear to have suffered a detrimental physiological effect, there was the question of whether or not the dogs would respond differently to physiological stress. In these studies the animals were stressed through a reduction in blood volume of each dog to 50% of its original value through phlebotomy.

The recuperation from this insult appeared to be identical for the three groups of dogs, and the curves reflecting the blood parameters could be essentially superimposed. The bleeding procedure did not seem to influence the blood lead level of the animals, which returned to the prephlebotomy level some one week after the loss of blood.

In the study of hemoglobin synthesis a convenient biological model is available in cultures of the photosynthetic microorganism, Rhodopseudomonas spheroides. This organism grows very well, as a facultative anaerobe, without oxygen, in which case it produces bacteriochlorophyll, or in the presence of oxygen, it will grow in the dark and manufacture primarily heme. Lead suppresses the growth of the organism very markedly when the culture is relatively low in iron. When supplies of iron are sufficient, the inhibitory effect of lead on the growth of the organism is much less apparent.

The excretion level of coproporphyrin by this organism clearly demonstrates the antagonism between iron on the one hand and lead, manganese, and cobalt on the other. When the organism is grown at low concentrations of iron and in the absence of other metals very little coproporphyrin is excreted. The addition of 0.03 ppm manganese to the culture medium, however, causes the excretion of large amounts of coproporphyrin. In iron-supplemented cultures much higher quantities of manganese are tolerated before the excess coproporphyrin appears. Lead potentiates the effect of manganese in causing increased coproporphyrin excretion and is thus antagonistic to the addition of iron. If then still more iron is added to the medium one overcomes the effect of manganese entirely and the effect of lead to a somewhat lesser extent. In all instances the effect of a metal such as lead can only be stated in terms of the relative concentration of other metals which act at the same binding site.

An examination of the other metabolites in the tetrapyrrole synthetic pathway suggests that the enzyme coproporphyrinogen oxidase or coproginase may be iron-dependent in that its activities are influenced by the balance between iron and other antagonistic metals including lead. Ferrochelatase and ALA dehydrase may also be affected by other metals as well as lead. In the whole growing organism the coproginase site seems to be a most important area for lead inhibition to be manifest, and this may well provide a basis for the well-known coproporphyrinuria of plumbism in man.

V. Summary of Reports on Lead and the Nervous System

Clinical manifestations caused by excessive amounts of systemic lead depend upon the particular structures which are affected or damaged and the intensity and duration of exposure. Levels of lead in blood and urine may reflect the current intensity of exposure. The actual lead burden, however, must also include an unmeasured quantity of lead stored in the central and peripheral nervous systems as well as in the skeleton and soft tissues. The persisting effects of encephalopathy, such as mental retardation, epilepsy, and behavioral disorders, may be the result of anatomical damage occurring during the acute intoxication or possibly the result of chronic cellular damage resulting from the slow release of stored lead in combination with continued exogenous exposure. Experienced observers have repeatedly emphasized the importance of continued environmental exposure to lead as an important factor which increases the likelihood of severe permanent damage to the brain.

An additional hypothesis is that continued exposure by the slow release of stored amounts of lead, which in themselves do not produce obvious symptoms may, nevertheless, be capable of damaging certain intracellular enzyme systems and producing latent effects. The concept of latent effects due to lead in persons who do not show obvious clinical plumbism has been raised by Lane and others who believe that lead burdens insufficient to cause symptoms or to produce disability may shorten life expectancies because of premature development of nephritis or cerebral hemorrhage. The evidence for this is not firm, however.

The neurological manifestations of lead poisoning are variable and widespread. Lead workers may complain of lassitude, irritability, depression, constipation, and abdominal discomfort. These symptoms are the same as those sometimes expressed by emotionally depressed individuals.

Peripheral neuropathy is considered a common neurologic manifestation of lead poisoning, and involvement of the radial nerve is frequently described. While the clinical picture may present as a mononeuropathy, neurological examination will often demonstrate weakness in other muscles, and neuroelectrical studies may show prolonged conduction times or decreases in the amplitudes of the action potential. In some cases, spastic paraplegia and hyperreflexia suggest upper arc and lower neuron damage. In such cases the differential diagnosis between lead neuromyelopathy and spinal cord degeneration such as in primary lateral sclerosis and amyotrophic lateral sclerosis may be difficult.

Lead encepholopathy has been considered more common in children than in adults, in whom it may be misdiagnosed as a brain tumor responsible for such symptoms as headache, vomiting, diplopia, and periodic confusion, in conjunction with evidence of increased intracranial pressure. Ophthalmological disturbances are not common in systemic lead poisoning although lead pigmentation of the retina has been described in adults. Ocular symptoms of lead poisoning have been more common in children in whom it is possible to recognize disturbances of the visual cortex, suprageniculate pathways, optic nerve, retina, lens, and intra- and extra-ocular muscle movement.

Of the neurologic manifestations of lead poisoning in children, peripheral neuropathies have not been the most apparent, and central nervous system manifestations have been considered more common. It is possible, however, that examples of peripheral neuropathy in children go unrecognized in many cases, including those in whom encephalopathy is dramatic and commands clinical attention. Children with clinical evidence of peripheral neuropathy, viz., sensory loss and weakness show evidence of denervation when tested electrically.

Neurochemical studies of the brain in lead poisoning have been technically difficult and have most commonly focused upon the effect of alkyl lead compounds, the pharmacology for which is undoubtedly dissimilar to that for inorganic lead. Seizures encountered in acute lead encephalopathy may be due to neuronal excitation related to local edema or vascular lesions. These manifestations may be due to an interference with glutamic acid transport or metabolism resulting in a reduction of γ-aminobutyric acid (GABA) production. GABA has been considered an inhibitory transmitter or depressant moderator, and interference with GABA production results in seizures by increasing neuronal excitability. Metabolic steps which might be susceptible to the effects of lead include (1) glutamic acid decarboxylase (GAD) which carboxylates glutamate to GABA; (2) glutamic acid dehydrogenase (GHD) which reduces 2-keto glutarate to glutamic acid; and (3) pyridoxal phosphate (B_6P) metabolized from pyridoxine which functions as the co-enzyme for transamination reactions in the synthesis of GABA. Further evaluation of these systems and of the clinical efficacy of pyridoxine or glutamate in children with seizures of acute lead poisoning is warranted.

Recent electron microscopic studies in experimental lead neuropathy have shown that the primary damage is to Schwann cells and myelin sheaths but that axons also show degenerative and reactive changes. Steps in the remyelination process

have been demonstrated microscopically. Two mechanisms have been suggested in the explanation of the process of demyelination and remyelination in lead neuropathy. One possibility is that a substance having porphobilinogen as a precursor is essential for the maintenance of myelin. It is known that in porphyria there is a metabolic block, which results in the excretion of excess porphobilinogen and d-ALA and concurrently a demyelinating process, the specific cause of which is not known. The second possibility is that lead causes vasodilation and altered vascular permeability which cause intra-endoneural edema and consequent damage to the Schwann cells by pressure or ischemia.

Biochemical studies of lead neuropathy have concentrated on the motor endplate region of striated muscles, the point where the greatest proportion of synaptic acetylcholinesterase is localized. The introduction of lead into suitable neuromuscular preparations results in the precipitation of lead at the esteratic site of this enzyme. The prime binding site for lead appears to be the post-synaptic subneural apparatus of the motor endplate. The binding of the subneural apparatus by lead is not influenced by the prior administration of di-isopropyl fluorophosphate (DFP) or neostigmine. However, this type of treatment completely inhibits cholinesterase activity. Cholinesterase activity does appear to be reduced in muscle in which the subneural apparatus has been bound by lead. Such studies are part of an attempt to identify some properties of the cholinergic receptor and the characteristics of lead binding at the motor endplate which may be related to some aspects of the weakness found in lead poisoning.

Other studies have focused upon calcium, which is normally present at the motor endplate in a bound form and is released as the free ion following nerve stimulation. The close relationship between the site of calcium release and the site of binding of divalent metal ions such as lead may explain the predilection for palsy in the more active muscles. Lead and calcium may also be interrelated through a mechanism which is reflected in the fact that a reduction in calcium concentration may produce neuromuscular blockade by decreasing the presynaptic release of acetycholine.

VI. Conference Summary

This conference reaffirmed a long known fact that poisonings due to moonshine liquor containing lead, pica for

lead-containing material, and excessive occupational exposure to lead continue to occur. This fact represents a failure on the part of health experts and other responsible elements of society to prevent gross exposures to lead.

The presentations of this conference illustrate the increasingly sophisticated methodology being used to study the manifestations of excessive lead absorption. Such studies are obviously important to our understanding of the mechanisms and consequences of lead intoxication. Of even greater importance, however, would be the development of sensitive and specific measures which could establish whether or not "normal" people are being harmed by current concentrations of lead in the "normal" environment. No new, practical measures are as yet available as screening procedures to detect very early adverse responses to lead. The validity of new enzyme studies is currently under examination.

Aminoaciduria. Abnormal quantities of aminoacids in urine as a consequence of lead exposure occur only in people with overt lead poisoning. Barely elevated levels of aminoacids can be detected in urine of workers with excess lead exposure. Aminoaciduria, therefore, does not appear to be a highly sensitive clinical study procedure for "normal" populations, although it is certainly important for research in cases of lead intoxication.

Inclusion Bodies. Nuclear inclusion bodies in kidney cells appear to be relatively specific but to date have only been found after relatively high exposures to lead. Their only presently known function is as a storage site for excess lead which has entered the nucleus of kidney cells and complexed with mitochondria. Intranuclear inclusion bodies should be looked for in cadaver studies, particularly in those with high body burdens of lead, and in cells in the urinary sediment of humans with chronic lead exposure. In addition various animal species should be used in laboratory studies of nuclear inclusion bodies.

"Sodium Pump" Inhibition. This phenomenon presents as a hypothetical lead effect and requires research for verification.

Biochemical Functions. Two potentially useful indicators of effect of lead are available: the measurement of the respiration of mitochondria of reticulocytes and the measurement of potassium loss across the red blood cell membrane. Both of these phenomena require evaluation in animals and humans with lead intoxication.

Enzyme Changes. Enzymes which are considered to be most sensitive to lead exposure are d-aminolevulinic acid dehydrase, lipoamide dehydrogenase and ATP-ase. Changes in enzyme levels need to be correlated with possible adverse effects in man and animals.

Essential Trace Metals. Many trace metals are essential for normal body functions, and lead may be competitive with these metals. However there are no known examples of such inhibition by lead in *in vivo* systems. Cadaver studies have shown that essential trace metals in soft tissues are normal despite increased concentrations of lead.

Heme Synthesis. Coproporphyrin and delta amino levulinic acid in urine are sensitive and reasonably specific indicators of lead exposure. These measurements are of value in studies with adults, but may be of less value in studies with children. There are marked diurnal variations in these indicators, and it is recommended that concentrations be expressed in units relative to urine output per unit time rather than to urine volume. Delta amino levulinic acid determinations in serum are suggested as a more refined measure of lead effect upon heme synthesis. The behavior of ALA-dehydrase under lead exposure situations requires examination and interpretation from the point of view of health.

Nerve Conduction Time. One of the more practical tests for subacute and chronic neurological change is the measurement of nerve conduction time. This procedure is considered to be more useful for following cases of lead intoxication than for screening purposes although lead poisoning should be considered in the presence of unexplained delayed conduction times.

Analytical Variability. For almost every study, questions about analytical accuracy and precision can be raised. It is suggested that comparisons of data would be facilitated by collaboration through a central laboratory. This technique would avoid much controversy and expedite interpretations of data.

This conference reviewed the state of current knowledge about how lead interacts with man and experimental living systems. It appears that the best indicators of early response to lead are already in use. Several areas of research which may lead to improved measures of early response are apparent.

It has been reassuring to note that exhaustive animal studies have not led to the discovery of new lesions due to lead, although it is also clear that there are major differences between species in response to lead absorption.

Several needs for the immediate future are highlighted.

1. Prevention of excessive and unusual exposures to lead--a broad problem for society-at-large.

2. Refined studies of those cases of lead intoxication which, unfortunately, continue to occur.

3. Refined studies on populations with elevated, but not necessarily clearly harmful, exposures to lead, especially with respect to changes in renal function and heme synthesis.

4. Research with animals on correlations between enzyme changes and adverse effects of lead.

5. Surveillance of the environment and populations for continuing evidence of man's existing and previous exposure to lead.

6. Surveillance for potentially susceptible individuals and indications of synergistic actions or aggravation of existing disease.